Final Report

In vitro **Mammalian chromosome aberration test of MWCNT using cultured Chinese Hamster Ovary (CHO-k1) cells**

(Study No. : GT13-00018)

November 2014

the way to trust KCL Korea Conformity Laboratories

Bioconvergence Technology Laboratory

Statement

Study No. : GT13-00018

Title : *In vitro* Mammalian chromosome aberration test of MWCNT using cultured Chinese Hamster Ovary (CHO-k1) cells

This final report was written in Korean and translated into English.

This study has been performed in compliance with the principles of Good Laboratory Practices and test guidelines in following documents.

1. National Institute of Environment Research (NIER) [Notice No. 2012-23, (revised 22 August 2012)].
2. OECD Guideline for Testing of Chemicals No. 473 '*In vitro* Mammalian chromosome aberration test'(Adopted: 21 July 1997)

The stated object in study protocol was achieved and there were no significant deviations from the aforementioned regulations that affected quality or integrity of the study. Therefore the justification of all data in this study was confirmed. The information of the test substance was written from the document that study sponsor provided.

14 November 2014

Jin-Sik Kim
Study Director
Bioconvergence Technology Laboratory, Korea Conformity Laboratories

Date

Nov. 14. 2014

Jin-Kyu Lee
Management
Bioconvergence Technology Laboratory, Korea Conformity Laboratories

Date

QUALITY ASSURANCE STATEMENT

Study No. : GT13-00018

Title : *In vitro* Mammalian chromosome aberration test of MWCNT using cultured Chinese Hamster Ovary (CHO-k1) cells

This study was subject to audit by the independent Quality Assurance Unit of KCL as indicated below. The findings of each audit were reported to the study director and management as prescribed by Standard Operating Procedures.

The final report audit was designed to confirm that as far as can be reasonably established the methods described and results incorporated in the final report accurately reflect the raw data produced during the study.

Audit phases and dates reported to the responsible personnel were as indicated below and these were based upon the audit records.

Phase Inspected	Date	Reports to Study Director	Reports to Management
Study Plan	2013. 02. 15	2013. 02. 15	2013. 02. 15
Storage of test substance and vehicle	2013. 02. 28	2013. 02. 28	2013. 02. 28
Strains(cell)	2013. 02. 28	2013. 02. 28	2013. 02. 28
Preparation of media and inoculation of cell	2013. 03. 04	2013. 03. 04	2013. 03. 04
Preparation of test substance	2013. 03. 07	2013. 03. 07	2013. 03. 07
Treatment of substance	2013. 03. 07	2013. 03. 07	2013. 03. 07
Preparation of specimen and microscopic examination	2013. 03. 11	2013. 03. 11	2013. 03. 11
Raw data	2013. 08. 07	2013. 08. 07	2013. 08. 07
Final report	2013. 08. 07	2013. 08. 07	2013. 08. 07

QA director : Song, Kyung Seuk Ph.D. Date 2013. 08. 07.
Auditor, Quality Assurance

* signed original

Study Personnel

Sample preparation	Jae-Hyuck Sung *	**Date**	07 August 2013
Techincal Assistant	Hyo-Jin Joo *	**Date**	07 August 2013
Cell preparation	Jin-Sik Kim *	**Date**	07 August 2013
Archiving of documents	Hyo-Dong Kim *	**Date**	07 August 2013

* Signed original

Title	*In vitro* Mammalian chromosome aberration test of MWCNT using cultured Chinese Hamster Ovary (CHO-k1) cells	
Objectives	This test was performed to assess the ability of MWCNT to induce chromosomal aberrations in CHO-k1 cells.	
Sponsor	Name	: Bioconvergence Technology Laboratory, Korea Conformity Laboratories
	Client	: Jin-Kyu Lee
	Address	: 8, Gaetbeol-ro 145 beon-gil, Yeonsu-gu, Incheon, Korea
	Tel.	: 032-859-4041 Fax : 032-858-0020
Testing facility	Name	: Bioconvergence Technology Laboratory, Korea Conformity Laboratories
	Address	: 8, Gaetbeol-ro 145 beon-gil, Yeonsu-gu, Incheon, Korea
	Tel.	: 032-858-0017 Fax : 032-858-0020
Study Schedule	Study initiation	: 15 February 2013
	Cell seeding	: 04 March 2013
	Chemical treatment	: 07 March 2013
	Slide preparation	: 08 March 2013
	Submission of final report	: 07 August 2013

Archives

1) Period of storage : 5 years after study completion
2) Documents of storage : Study plan, Documents related to the test substance, Raw data, Final report, Documents related to the GLP
3) Room of specimen storage : Slides
4) Room of storage : CD, Related documents

Contents

1. Summary ····· 1

2. Test substance and control substances
1) Test substance ····· 2
2) Negative control (Solvent 1) ····· 2
3) Negative control (Solvent 2) ····· 2
4) Negative control (Solvent 3) ····· 2
5) Negative control (Solvent 4) ····· 3
6) Positive control substance Ⅰ (Without metabolic activation) ····· 3
7) Positive control substance Ⅱ (With metabolic activation) ····· 3
8) Justification for the selection of control substances ····· 4

3. Materials and methods
1) Test system ····· 4
2) Metabolic activation system (S9 mix) ····· 5
3) Test method ····· 5
(1) Preparation of test substance ····· 5
(2) Preliminary range-finding test ····· 6
(3) Main test ····· 6
4) Evaluation of metaphase and statistical analysis ····· 7
(1) Observation method ····· 7
(2) Observation criteria ····· 7
(3) Statistical analysis and evaluation ····· 7

4. Results
1) Preliminary range-finding test ····· 9
2) Main test ····· 9

5. Discussion and conclusion ····· 11

6. References ····· 12

7. Figures & Tables (Group summary)
Figure 1. The number of cells with chromosome aberrations in the absence of S9 mix

(24 hrs treatment). ····· 13
Figure 2. The number of cells with chromosome aberrations in the absence of S9 mix (6 hrs treatment). ····· 13
Figure 3. The number of cells with chromosome aberrations in the presence of S9 mix (6 hrs treatment) ····· 14
Table 1. The number of cells with chromosome aberrations in the absence of S9 mix ····· 15
Table 2. The number of cells with chromosome aberrations in the presence of S9 mix ····· 16

8. Appendices (Individual data)
Appendix 1. The number of cells with chromosome aberrations in the absence of S9 mix ····· 17
Appendix 2. The number of cells with chromosome aberrations in the presence of S9 mix ····· 18

9. Annexes
Annex 1. Test result of relative cell count (preliminary range-finding test) ····· 19
Annex 2. Test substance data sheet ····· 20
Annex 3. Quality control statement of S9 ····· 21
Annex 4. GLP certificate ····· 22
Annex 5. Quality assurance statement-Original ····· 25
Annex 6. Study personnel-Original ····· 26

1. Summary

The clastogenicity of MWCNT was examined in chromosome aberration test using cultured Chinese Hamster Ovary (CHO-k1) cells in both the presence and absence of metabolic activation system (S9 mix). The test substance was dispersed in DPPC solution and serially diluted with DPPC solution.

In order to assess the cytotoxicity of the test substance in cultured CHO-k1 cells, Relative Cell Count (RCC) was calculated for all cultures treated with the test and control substances. On the basis of this preliminary test, the following treatment times and concentrations were selected for the main study.

- 24 hours continuous treatment (without S9 mix) :
  0.78, 1.56, 3.13 ㎍/㎖
- 6 hours treatment and 18 hours recovery (without S9 mix) :
  0.78, 1.56, 3.13 ㎍/㎖
- 6 hours treatment and 18 hours recovery (with S9 mix) :
  0.78, 1.56, 3.13 ㎍/㎖

As a result of the main test, the test substance did not produce a statistically significant increase in the number of cells with chromosome aberrations at all dose levels when compared with the negative control in the absence of S9 mix (24 hours continuous treatment group and 6 hours treatment and 18 hours recovery group).

In the presence of S9 mix (6 hours treatment and 18 hours recovery), the test substance caused no statistically significant increase in the number of cells with chromosome aberrations at all dose levels when compared with negative control.

Furthermore, the test substance did not induce a statistically significant increase in the number of cells with polyploidy or endoreduplication when compared with the negative control in the presence and absence of metabolic activation system (S9 mix).

Based on the above results, it is concluded that the test substance MWCNT is not capable of inducing chromosome aberration in cultured CHO-k1 cells under the condition of this study.

2. Test substance and control substances

1) Test substance

(1) Product name : MWCNT (KUMHO: K-Nanos-100P)

(2) Lot No. : Not available

(3) Received date : 25 January, 2013

(4) Received quantity : 666.89 g (including container weight)

(5) Appearance : Powder

(6) Purity : More than 90 % (We assumed 100 % for test substance and carried out the test)

(7) Solubility : The test substance was dispersed in DPPC solution at 1.0 % concentration.

(8) Storage condition : Room temperature

(9) Stability : Not available

(10) Caution : Not available

(11) Supplier : KUMHO PETROCHEMICAL

2) Negative control substance (solvent 1)

(1) Name : 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)

(2) Lot No. : 078K5203

(3) CAS No. : 63-89-8

(4) Molecular weight : 734.04

(5) Date received : 21 March 2012

(6) Quantity Received : 1 g

(7) Appearance : White powder

(8) Grade : ≥ 99 %

(9) Storage condition : Freezing storage

(10) Supplier : Sigma-Aldrich, Inc.

3) Negative control substance (solvent 2)

(1) Name : Dulbecco's phosphate buffered saline (DPBS)

(2) Lot No. : 031M8307

(3) CAS No. : Not available

(4) Date received : 08 May 2012

(5) Quantity Received : 480 g

(6) Appearance : White powder

(7) Storage condition : Cold storage

(8) Supplier : Sigma-Aldrich, Inc.

4) Negative control substance (solvent 3)

(1) Name : D-(+)-Glucose
(2) Lot No. : 071M0145V
(3) CAS No. : 50-99-7
(4) Date received : 28 August 2012
(5) Quantity Received : 1 ㎏
(6) Appearance : White powder
(7) Storage condition : Room temperature
(8) Supplier : Sigma-Aldrich, Inc.

5) Negative control substance (solvent 4)
(1) Name : Bovine serum albumin
(2) Lot No. : 750462
(3) CAS No. : Not available
(4) Date received : 06 April 2009
(5) Quantity Received : 100 g
(6) Appearance : Brown powder
(7) Storage condition : Cold storage
(8) Supplier : Gibco

6) Positive control substance I (Without metabolic activation)
(1) Name : Mitomycin C (MMC)
(2) Lot No. : 010M0665
(3) CAS No. : 50-07-7
(4) Molecular weight : 334.3
(5) Date received : 09 July 2012
(6) Quantity received : 2 mg
(7) Feature : Water soluble
(8) Storage condition : Cold storage (4 ℃)
(9) Supplier : Sigma-Aldrich, Inc.
(10) Concentration : 0.04 ㎍/㎕

7) Positive control substance Ⅱ (with metabolic activation)
(1) Name : Cytophosphamide · H_2O (CPA)
(2) Lot No. : 120M1253
(3) CAS No. : 6055-19-2
(4) Molecular weight : 279.1
(5) Date received : 28 March 2011
(6) Quantity received : 1 g
(7) Feature : Water soluble
(8) Storage condition : Cold storage (4 ℃)

(9) Supplier : Sigma-Aldrich, Inc.

(10) Concentration : 10 ㎍/㎕

8) Justification for the selection of control substances

Prior to execution of this study, the solubility of the test substance was not well-dispersed in general dispersion agents. For this reason, we selected the DPPC solution (5.5 mM D-(+)-glucose + 0.6 mg/ml Bovine serum albumin + 0.01 mg/kg DPPC in DPBS) according to Kim et al., 2011 study (Evaluation of biocompatible dispersants for carbon nanotube toxicity tests, Arch. Toxicol. 85: 1499-1508). As a result of solubility test, we observed that the test substance was well-dispersed in DPPC solution at 1.0 % concentration.

Positive control substances were selected according to OECD guidelines No. 473.

3. Materials and methods

1) Test system

This study was performed with Chinese Hamster Ovary cell (CHO-k1) that was obtained from Korean Cell Line Bank (KCLB).

(1) Justification for selection of cell line

We selected CHO-k1 cells which were known for high sensitivity to chemicals and have much study data in chromosome aberration studies.

(2) Method of cultivation

① Culture medium : F-12 Nutrient Mixture (GIBCO, Lot No. 1237575) with 10% Fetal Bovine Serum (Hyclone, Lot No. AXC36539).

② Culture condition : Incubator maintained at 5 % CO_2, 37 ℃. Subculture was conducted every 3～4 days.

③ Doubling time : about 15 hours

④ Modal chromosome number : 22

⑤ Storage condition : Cells were cryopreserved in F-12 Nutrient Mixture with 10 % Fetal Bovine Serum (FBS) and 10 % DMSO and stored in liquid nitrogen (-196 ℃).

2) Metabolic activation system (S9 mix)

(1) S9

Supplier	Molecular Toxicology Inc.
Manufacture date	20 September 2012
Date of acquisition	01 December 2012
Expiry Date	20 September 2014
Lot No.	3003
Storage condition	-80℃ (Deep Freezer DF9007)
Protein content	39.5 ㎎/㎖

(2) Preparation of S9

Animal		Inducing agent	
Species	SD Rat	Agent	Aroclor 1254 (Monsanto Lot No. KL615)
Sex	Male	Route	I.P.
Age	7 weeks	Buffer	0.154 M KCl

(3) S9 Mix

Ingradients	Concentration
S9	0.3 ㎖
$MgCl_2$	5 μmol
KCl	33 μmol
G-6-P	5 μmol
NADP	4 μmol
HEPES buffer	4 μmol
Distilled water	-

3) Test method

This study was conducted in accordance with the following test guidelines:

National Institute of Environment Research (NIER) [Notice No. 2012-23, (revised 22 August 2012)].

OECD Guideline for Testing of Chemicals No. 473 'In vitro Mammalian chromosome aberration test'(Adopted: 21 July 1997).

The standard operation procedures (SOPs) of Korea Conformity Laboratories (KCL/CRO).

(1) Preparation of test substance

The test substance was dispersed in DPPC solution and serially diluted with DPPC solution.

(2) Preliminary range-finding test

In order to determine the cytotoxicity and dosing concentration for the main test, the preliminary range-finding test was performed. The cultured cells were treated with the following 8 concentrations (1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200 ㎍/㎖) of test substance both with and without S9 mix along with control substances. The number of cells per culture dish was calculated for each concentration by hemacytometer. Relative cell count (RCC) was calculated according to the following formula : RCC (Relative Cell Count) = (No. of treated cells/No. of control cells)×100 (%)

(3) Main test

The main test was conducted at 3 concentrations determined by the preliminary range-finding study. Two replicate cultures were used for each concentration level.

① Without S9 mix (6 hours treatment and 24 hours treatment)

Based on the cell count, test cultures containing 4×10^4 cells/㎖ were seeded in 60 mm diameter tissue culture dishes and incubated for 3 days. The culture medium was removed from the culture dish and 4.90 ㎖ of pre-warmed fresh medium was added to each dish. Then, 0.10 ㎖ of test substance (MWCNT) was added to each dish.

For 6 hours treatment and 18 hours recovery group, the cells were exposed to the test substance for 6 hours. The culture medium was removed and the cells were rinsed with PBS (Ca^{2+} & Mg^{2+} free Dulbecco's phosphate buffered saline). 5 ㎖ of complete culture medium was added and the culture was incubated for an additional 18 hours.

② With S9 mix (6 hours treatment)

Based on the cell count, test cultures containing 4×10^4 cells/㎖ were seeded in 60 mm diameter tissue culture dishes and incubated for 3 days. The culture medium was removed from culture dishes and 4.40 ㎖ of pre-warmed fresh medium was added to each dish. 0.10 ㎖ of test substance (MWCNT) and 0.50 ㎖ of S9 mix was added to each dish. The cells were exposed to the test substance for 6 hours. The culture medium was removed and cells rinsed with PBS (Ca^{2+} & Mg^{2+} free Dulbecco's phosphate buffered saline). 5 ㎖ of complete culture medium was added and then the culture was incubated for an additional 18 hours.

③ Slide preparation

Approximately 22 hours after treatment, Colcemid (GIBCO, 1150757) was added to each culture for a final concentration of 0.2 ㎍/㎖. The cultures were incubated for an additional 2 hours. The cells were detached using 1× trypsin solution. The medium containing mitotic cells was centrifuged at 1,000 rpm for 5 minutes, and the cell pellets were resuspended in 75 mM potassium chloride (KCl) solution. After 20

minutes at room temperature, the cells were fixed 3 times with Carnoy's fixative solution (methanol : glacial acetic acid = 3 : 1 v/v). Two slides were prepared from each fixed cell suspension. The slides were air-dried, stained with 5% Giemsa (Merck, HX105037) for 5 minutes and observed microscopically.

4) Evaluation of metaphase and statistical analysis

(1) Observation method

The analysis was conducted at criteria of 100 metaphase cells per plate as described below. The frequency of aberration is the ratio of normal cells to cells exhibiting chromosomal aberration. Blinded slide observation was performed.

① Structural aberration

Gap (Chromatid type, Chromosome type) : gap

Breakage (Chromatid type) : ctb

Exchange (Chromatid type) : cte

Breakage (Chromosome type) : csb

Exchange (Chromosome type) : cse

② Numerical aberration

Numerical aberration : PP, ER

(2) Observation criteria

① Gap : Gap is an achromatic lesion smaller than the width of one chromatid and with minimum chromatid misalignment.

② Breakage : Breakage is an achromatic lesion larger than the width of 2 folds of one chromatid. It is as far away from alignment and does not have kinetochore.

③ Exchange : DNA breaks (in one or more chromosome) can participate in the production of aberrations. Broken free ends in neighbouring chromosomes can interact and form DNA exchanges.

④ Others : Fragmentation includes many gaps and breakages but without exchanges.

⑤ Numerical aberration : CHO-k1 has a modalchromosome number of 22. Anueploidy is not counted as numerical aberration since it occurs frequently. Thus, only polyploid is classified as a numerical aberration (over 3n=36) Endoreduplication was also recorded.

(3) Statistical analysis and evaluation

The number of aberrant metaphases, excluding gaps (according to OECD guideline), and number of (Polyploid + Endoreduplication) were analyzed. The statistical analysis were performed with SPSS 12.1K program. The result of statistical evaluation was regarded as significant when the p value was less than 0.05.

① The negative control and treated groups received a Chi-square test.

② The negative and positive control groups received a separate Chi-square test

③ Linear logistic regression test was performed for dose-response.

④ Study evaluation: Study results were judged as positive if there was a dose-related and statistically significant increase in the number of aberrant metaphases, or if a reproducible positive results was detected in at least one test concentration.

4. Results

1) Preliminary range-finding test

In order to determine the treatment concentration of the main test, Relative Cell Count (RCC) was calculated for all cultures treated with the test substance and control substance at the 8 dose levels (1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200 ㎍/㎖).

In the 24 hours continuous treatment group (without S9 mix), RCCs were 43.87 % and 49.57 % in order of 6.25 ㎍/㎖ and 3.13 ㎍/㎖. In the 6 hours treatment and 18 hours recovery group (without S9 mix), RCCs were 42.69 % and 49.00 % in order of 6.25 ㎍/㎖ and 3.13 ㎍/㎖ (without S9 mix). Also, we observed test substance precipitation at 6.25, 12.5, 25, 50, 100, 200 ㎍/㎖ in the 24 and 6 hours treatment group (without S9 mix) (Annex 1).

In the 6 hours treatment and 18 hours recovery group (with S9 mix), RCCs were 44.42 % and 49.18 % in order of 6.25 ㎍/㎖ and 3.13 ㎍/㎖ (with S9 mix). We also observed test substance precipitation at 6.25, 12.5, 25, 50, 100, 200 ㎍/㎖ in the 6 hours treatment and 18 hours recovery group (with S9 mix) (Annex 1).

Considering RCC and test substance precipitation in the preliminary range-finding, the main test was conducted at the 3 concentration of 2 fold. The details of concentrations are following :

- 24 hours continuous treatment (without S9 mix) :
  0.78, 1.56, 3.13 ㎍/㎖
- 6 hours treatment and 18 hours recovery (without S9 mix) :
  0.78, 1.56, 3.13 ㎍/㎖
- 6 hours treatment and 18 hours recovery (with S9 mix) :
  0.78, 1.56, 3.13 ㎍/㎖

2) Main test

In the 24 hours continuous treatment group without S9 mix, the frequency of chromosome aberration is 0.5, 0.0, 1.0 and 0.5 at 0, 0.78, 1.56 and 3.13 ㎍/㎖. The test substance caused no statistically significant increase in the number of cells with chromosome aberration at all dose levels when compared with negative control group (Figure 1 & Table 1).

In the 6 hours treatment and 18 hour recovery group without S9 mix, the frequency of chromosome aberration is 0.0, 0.0, 0.5 and 1.0 at 0, 0.78, 1.56 and 3.13 ㎍/㎖. The test substance caused no statistically significant increase in the number of cells with chromosome aberration at all dose levels when compared with negative control group (Figure 2 & Table 1).
In the 6 hours treatment and 18 hours recovery group with S9 mix, the frequency of chromosome aberration is 0.0, 0.5, 0.5 and 0.5 at 0, 0.78, 1.56 and 3.13 ㎍/㎖. The test substance caused no statistically significant increase in the number of cells with chromosome aberration at all dose levels when compared with negative control group (Figure 3 & Table 2).

In the presence and absence of S9 mix, the test substance caused no statistically significant in the number of cells with polyploidy and endoreduplication, when compared with negative control group (Table 1 & 2).

5. Discussion and conclusion

This study was performed to assess the ability of MWCNT inducing chromosomal aberrations using cultured Chinese Hamster Ovary (CHO-k1) cells in both the presence and absence of metabolic activation system (S9 mix).

On the basis of preliminary range-finding test, the following treatment times and concentrations were selected for the main study.

According to main study result, we could not observe statistically significant increase in the number of cells with chromosome aberration at all dose levels when compared with negative control group in the 24 hours continuous treatment group (0.78, 1.56, 3.13 ㎍/㎖) and the 6 hours treatment and 18 hours recovery group (0.78, 1.56, 3.13 ㎍/㎖) without S9 mix (Figure 1 and 2 & Table 1).

In the 6 hours treatment and 18 hours recovery group (0.78, 1.56, 3.13 ㎍/㎖) with S9 mix, the test substance caused no statistically significant increase in the number of cells with chromosome aberration at all dose levels when compared with negative control group (Figure 3 & Table 2).

Based on the above results, it is concluded that the test substance MWCNT is not capable of inducing chromosome aberration in cultured CHO-k1 cells under the condition of this study.

6. References

1) National Institute of Environment Research (NIER) [Notice No. 2012-23, (revised 22 August 2012)].

2) Ishidate. M. Jr. (1987). Data book of chromosomal aberration test *in vitro*, revised edition, Life-Science Information Center, pp. 31-46.

3) JEMS-MMS (1998). Atlas of Chromosome aberration by chemicals, Japanese Environmental Mutagen Society-Mammalian Mutagenicity Study Group, Tokyo, Japan.

4) The Application of the Principles of GLP to *in vitro* Studies No. 14 (2004).

5) OECD (1997). OECD Guidelines for the Testing of Chemicals No. 473 '*In vitro* Mammalian chromosome aberration test' (Adopted : 21 July 1997)

7. Figures & Tables (Group Summary)

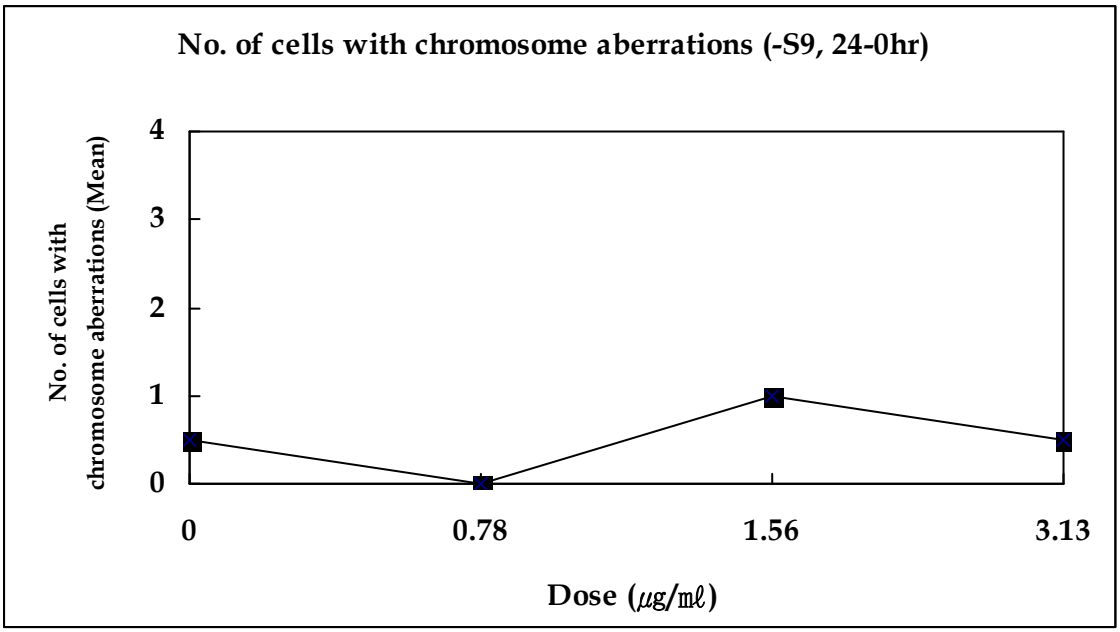


Figure 1. The number of cells with chromosome aberrations in the absence of S9 mix (24 hrs treatment).

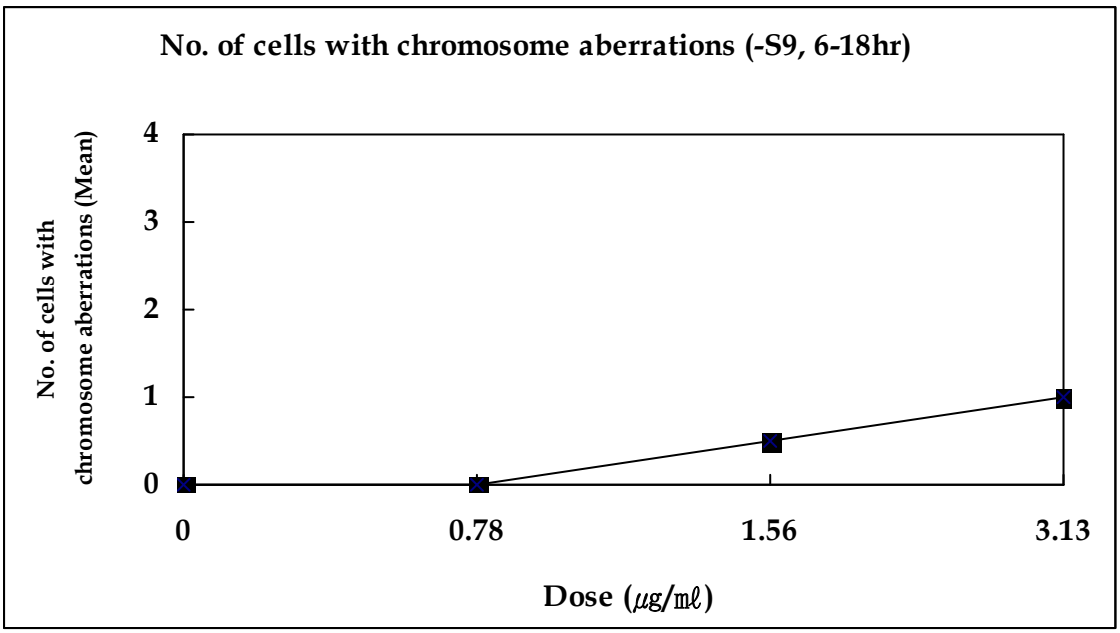


Figure 2. The number of cells with chromosome aberrations in the absence of S9 mix (6 hrs treatment).

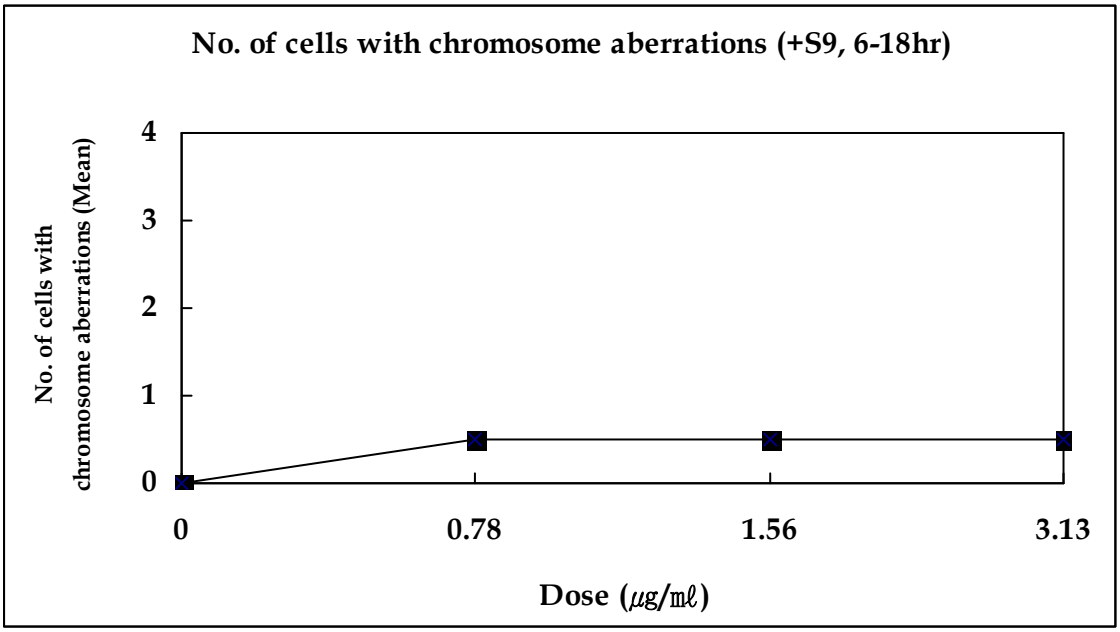


Figure 3. The number of cells with chromosome aberrations in the presence of S9 mix (6 hrs treatment).

Table 1. The number of cells with chromosome aberrations in the absence of S9 mix.

Exposure[a)]	S9 mix	Dose (μg/ml)	No. of total chromosome aberrations (Mean)		No. of cells with chromosome aberrations (Mean)		PP+ER (Mean)
			(-)Gap	(+)Gap	(-)Gap	(+)Gap	
24-0	-	Negative control	0.5	0.5	0.5	0.5	0
	-	0.78	0	0	0	0	0
	-	1.56	1	1	1	1	0
	-	3.13	0.5	0.5	0.5	0.5	0
	-	MMC (0.04)	28.5	29	28 *	28.5	0
6-18	-	Negative control	0	0	0	0	0
	-	0.78	0	0	0	0	0
	-	1.56	0.5	0.5	0.5	0.5	0
	-	3.13	1	1.5	1	1.5	0
	-	MMC (0.04)	24	24	24 *	24	0

* Significantly different from the negative control at $p<0.05$

Test substance : MWCNT

a) Treatment time-recovery time

MMC : Mitomycin C (0.04 μg/ml)
PP : Polyploidy
ER : Endoreduplication

Table 2. The number of cells with chromosome aberrations in the presence of S9 mix.

Exposure[a)]	S9 mix	Dose (μg/ml)	No. of total chromosome aberrations (Mean)		No. of cells with chromosome aberrations (Mean)		PP+ER (Mean)
			(-)Gap	(+)Gap	(-)Gap	(+)Gap	
6-18	+	Negative control	0	0	0	0	0
	+	0.78	0.5	0.5	0.5	0.5	0
	+	1.56	0.5	0.5	0.5	0.5	0
	+	3.13	0.5	0.5	0.5	0.5	0
	+	CPA (10)	25.5	25.5	25.5 *	25.5	0

* Significantly different from the negative control at $p<0.05$

Test substance : MWCNT

a) Treatment time-recovery time

CPA : Cyclophosphamide · H_2O (10 μg/ml)
PP : Polyploidy
ER : Endoreduplication

8. Appendices (Individual data)

Appendix 1. The number of cells with chromosome aberrations in the absence of S9 mix.

Exposure[a)]	Dose (μg/ml)	Cell No.	Aberration						No. of total chromosome aberrations		No. of cells with chromosome aberrations	
			Chromatid type		Chromosome type		PP/ER	Gap				
			ctb	cte	csb	cse			(-)Gap	(+)Gap	(-)Gap	(+)Gap
24-0	Negative control	100	0	0	0	0	0	0	0	0	0	0
		100	1	0	0	0	0	0	1	1	1	1
	0.78	100	0	0	0	0	0	0	0	0	0	0
		100	0	0	0	0	0	0	0	0	0	0
	1.56	100	1	1	0	0	0	0	2	2	2	2
		100	0	0	0	0	0	0	0	0	0	0
	3.13	100	0	0	0	0	0	0	0	0	0	0
		100	1	0	0	0	0	0	1	1	1	1
	MMC	100	3	25	0	0	0	0	28	28	28	28
		100	6	23	0	0	0	1	29	30	28	29
6-18	Negative control	100	0	0	0	0	0	0	0	0	0	0
		100	0	0	0	0	0	0	0	0	0	0
	0.78	100	0	0	0	0	0	0	0	0	0	0
		100	0	0	0	0	0	0	0	0	0	0
	1.56	100	0	1	0	0	0	0	1	1	1	1
		100	0	0	0	0	0	0	0	0	0	0
	3.13	100	1	0	0	0	0	1	1	2	1	2
		100	1	0	0	0	0	0	1	1	1	1
	MMC	100	3	22	0	0	0	0	25	25	25	25
		100	2	21	0	0	0	0	23	23	23	23

Test substance : MWCNT

a) Treatment time-recovery time

MMC : Mitomycin C (0.04 μg/ml)
PP : Polyploidy
ER : Endoreduplication

Appendix 2. The number of cells with chromosome aberrations in the presence of S9 mix.

Exposure[a)]	Dose (μg/ml)	Cell No.	Aberration						No. of total chromosome aberrations		No. of cells with chromosome aberrations	
			Chromatid type		Chromosome type		PP/ER	Gap				
			ctb	cte	csb	cse			(-)Gap	(+)Gap	(-)Gap	(+)Gap
6-18	Negative control	100	0	0	0	0	0	0	0	0	0	0
		100	0	0	0	0	0	0	0	0	0	0
	0.78	100	1	0	0	0	0	0	1	1	1	1
		100	0	0	0	0	0	0	0	0	0	0
	1.56	100	0	0	0	0	0	0	0	0	0	0
		100	0	1	0	0	0	0	1	1	1	1
	3.13	100	0	0	0	0	0	0	0	0	0	0
		100	0	1	0	0	0	0	1	1	1	1
	CPA	100	4	21	0	0	0	0	25	25	25	25
		100	3	23	0	0	0	0	26	26	26	26

Test substance :MWCNT

a) Treatment time-recovery time

CPA : Cyclophosphamide · H_2O (10 μg/ml)
PP : Polyploidy
ER : Endoreduplication

9. Annexes

Annex 1. Test result of relative cell count (preliminary range-finding test).

Exposure[a)]	S9 mix	Dose (μg/ml)	Cell counts (×10^6)					RCC(%)[b)]
			Plate A		Plate B		Mean	
24-0	-	N.C.	1.78	1.75	1.76	1.73	1.76	100.00
	-	1.56	0.97	0.93	1.04	1.08	1.01	57.26
	-	3.13	0.85	0.89	0.90	0.84	0.87	49.57
	-	6.25 †	0.76	0.74	0.78	0.80	0.77	43.87
	-	12.5 †	0.70	0.74	0.71	0.80	0.74	42.02
	-	25 †	0.71	0.77	0.80	0.68	0.74	42.17
	-	50 †	0.57	0.65	0.61	0.69	0.63	35.90
	-	100 †	0.66	0.51	0.62	0.67	0.62	35.04
	-	200 †	0.60	0.52	0.59	0.55	0.57	32.19
6-18	-	N.C.	1.70	1.73	1.76	1.79	1.75	100.00
	-	1.56	0.94	0.93	1.04	1.03	0.99	56.45
	-	3.13	0.92	0.85	0.85	0.80	0.86	49.00
	-	6.25 †	0.70	0.71	0.75	0.82	0.75	42.69
	-	12.5 †	0.53	0.61	0.63	0.62	0.60	34.24
	-	25 †	0.63	0.62	0.61	0.65	0.63	35.96
	-	50 †	0.54	0.50	0.55	0.52	0.53	30.23
	-	100 †	0.55	0.62	0.56	0.55	0.57	32.66
	-	200 †	0.58	0.54	0.56	0.54	0.56	31.81
6-18	+	N.C.	1.39	1.38	1.35	1.35	1.37	100.00
	+	1.56	0.79	0.77	0.87	0.81	0.81	59.23
	+	3.13	0.62	0.62	0.73	0.72	0.67	49.18
	+	6.25 †	0.63	0.62	0.57	0.61	0.61	44.42
	+	12.5 †	0.61	0.53	0.58	0.62	0.59	42.78
	+	25 †	0.40	0.47	0.47	0.52	0.47	34.00
	+	50 †	0.48	0.32	0.40	0.44	0.41	29.98
	+	100 †	0.32	0.41	0.32	0.30	0.34	24.68
	+	200 †	0.24	0.27	0.28	0.26	0.26	19.20

N.C. : Negative control

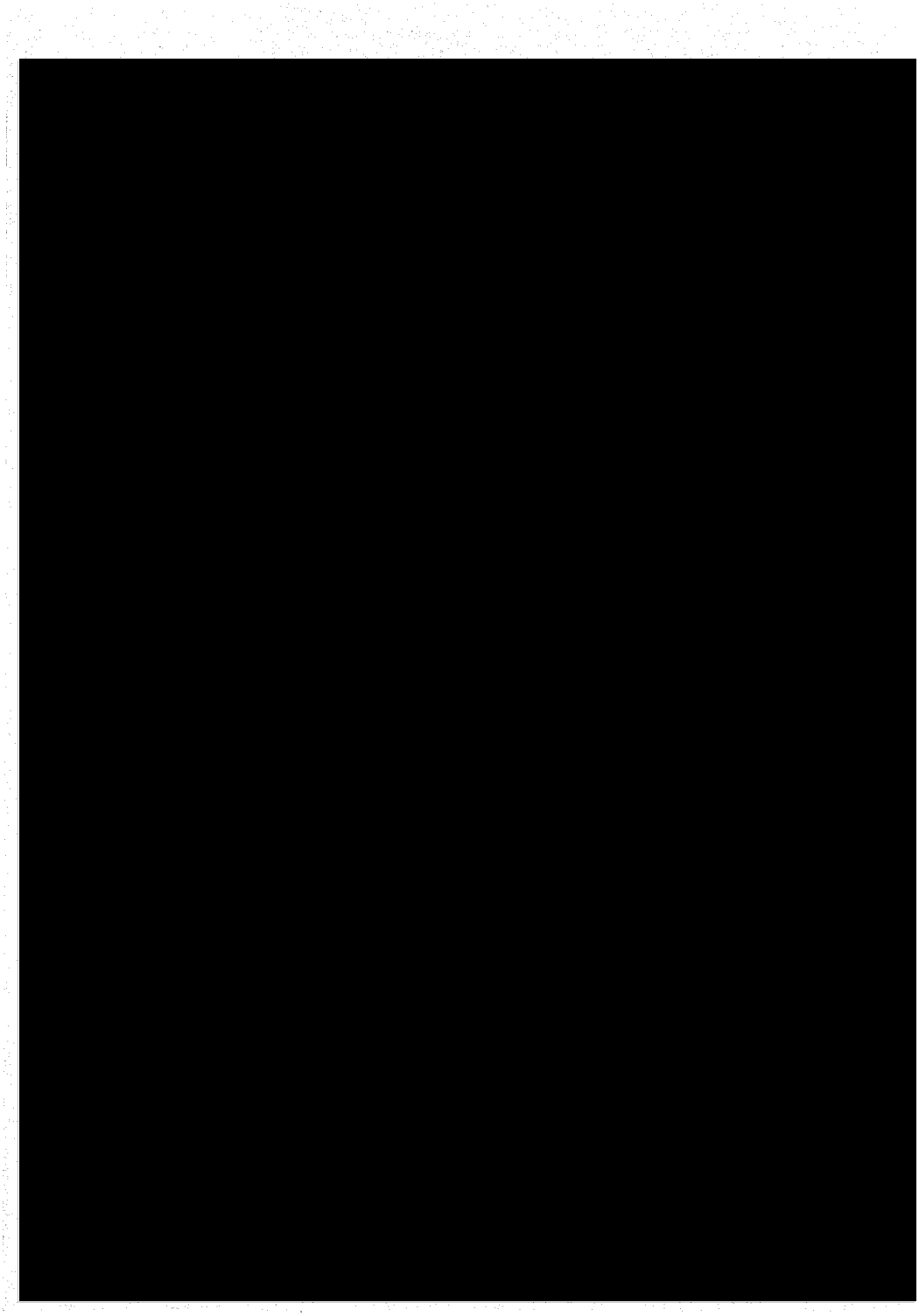
Test substance : MWCNT

a) Treatment time-recovery time

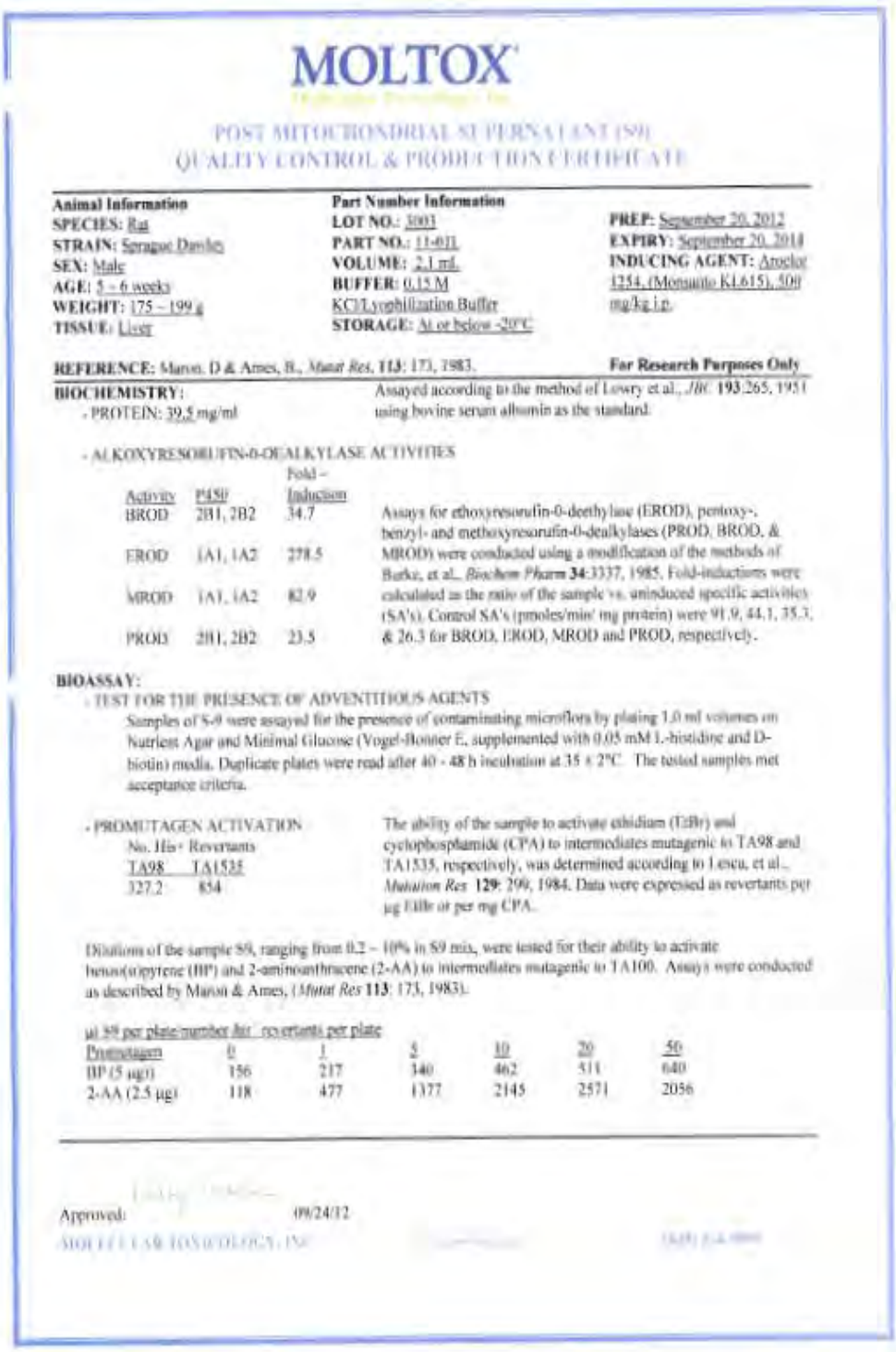
b) RCC (Relative cell count) = (No. of treated cells / No. of control cells) × 100 (%)

† : Test substance precipitation after treatment

Annex 2. Test substance data sheet



Annex 3. Quality control statement of S9



MOLTOX

POST MITOCHONDRIAL SUPERNATANT (S9)
QUALITY CONTROL & PRODUCTION CERTIFICATE

Animal Information
SPECIES: Rat
STRAIN: Sprague Dawley
SEX: Male
AGE: 5 – 6 weeks
WEIGHT: 175 – 199 g
TISSUE: Liver

Part Number Information
LOT NO.: 3003
PART NO.: 11-031
VOLUME: 2.1 mL
BUFFER: 0.15 M KCl/Lyophilization Buffer
STORAGE: At or below -20°C

PREP: September 20, 2012
EXPIRY: September 20, 2014
INDUCING AGENT: Aroclor 1254, (Monsanto KI.615), 500 mg/kg i.p.

REFERENCE: Maron, D & Ames, B., *Mutat Res*, **113**: 173, 1983.

For Research Purposes Only

BIOCHEMISTRY:

- PROTEIN: 39.5 mg/ml

Assayed according to the method of Lowry et al., *JBC* **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-O-DEALKYLASE ACTIVITIES

Activity	P450	Fold – Induction
BROD	2B1, 2B2	34.7
EROD	1A1, 1A2	278.5
MROD	1A1, 1A2	82.9
PROD	2B1, 2B2	23.5

Assays for ethoxyresorufin-O-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-O-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/ mg protein) were 91.9, 44.1, 35.3, & 26.3 for BROD, EROD, MROD and PROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Duplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ Revertants	
TA98	TA1535
327.2	854

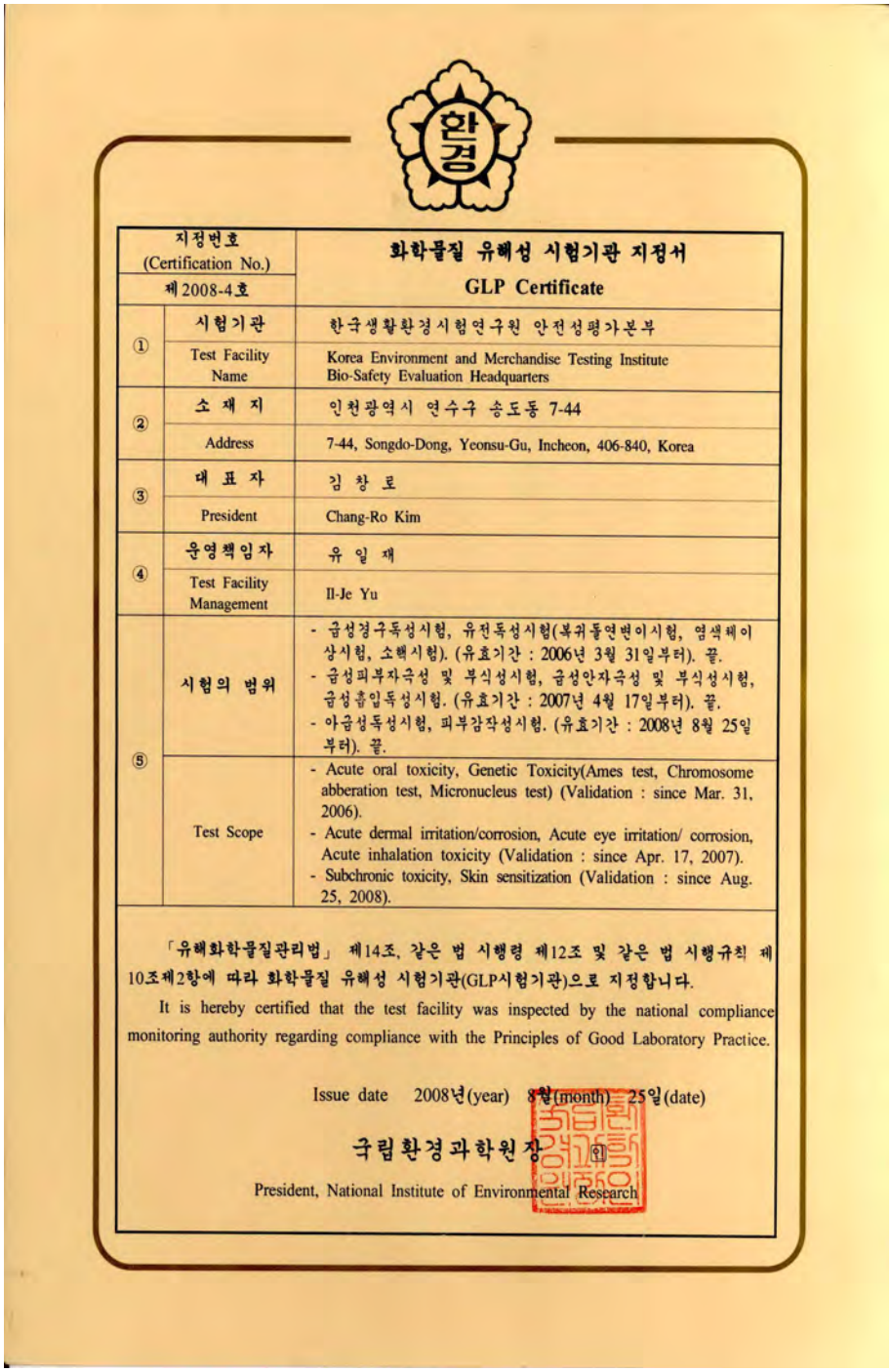
The ability of the sample to activate ethidium (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lescu, et al., *Mutation Res* **129**: 299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 – 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted as described by Maron & Ames, (*Mutat Res* **113**: 173, 1983).

µl S9 per plate/number his+ revertants per plate						
Promutagen	0	1	5	10	20	50
BP (5 µg/l)	156	217	340	462	511	640
2-AA (2.5 µg)	118	477	1377	2145	2571	2056

Approved: 09/24/12

Annex 4. GLP certificate



환경

지정번호 (Certification No.) 제2008-4호		화학물질 유해성 시험기관 지정서 GLP Certificate
①	시험기관	한국생활환경시험연구원 안전성평가본부
	Test Facility Name	Korea Environment and Merchandise Testing Institute Bio-Safety Evaluation Headquarters
②	소 재 지	인천광역시 연수구 송도동 7-44
	Address	7-44, Songdo-Dong, Yeonsu-Gu, Incheon, 406-840, Korea
③	대 표 자	김 창 로
	President	Chang-Ro Kim
④	운영책임자	유 일 재
	Test Facility Management	Il-Je Yu
⑤	시험의 범위	- 급성경구독성시험, 유전독성시험(복귀돌연변이시험, 염색체이상시험, 소핵시험). (유효기간 : 2006년 3월 31일부터). 끝. - 급성피부자극성 및 부식성시험, 급성안자극성 및 부식성시험, 급성흡입독성시험. (유효기간 : 2007년 4월 17일부터). 끝. - 아급성독성시험, 피부감작성시험. (유효기간 : 2008년 8월 25일부터). 끝.
	Test Scope	- Acute oral toxicity, Genetic Toxicity(Ames test, Chromosome abberation test, Micronucleus test) (Validation : since Mar. 31, 2006). - Acute dermal irritation/corrosion, Acute eye irritation/ corrosion, Acute inhalation toxicity (Validation : since Apr. 17, 2007). - Subchronic toxicity, Skin sensitization (Validation : since Aug. 25, 2008).

「유해화학물질관리법」 제14조, 같은 법 시행령 제12조 및 같은 법 시행규칙 제10조제2항에 따라 화학물질 유해성 시험기관(GLP시험기관)으로 지정합니다.

It is hereby certified that the test facility was inspected by the national compliance monitoring authority regarding compliance with the Principles of Good Laboratory Practice.

Issue date 2008년(year) 8월(month) 25일(date)

국립환경과학원장 [인]

President, National Institute of Environmental Research

(뒤 쪽)-1

<변경사항>

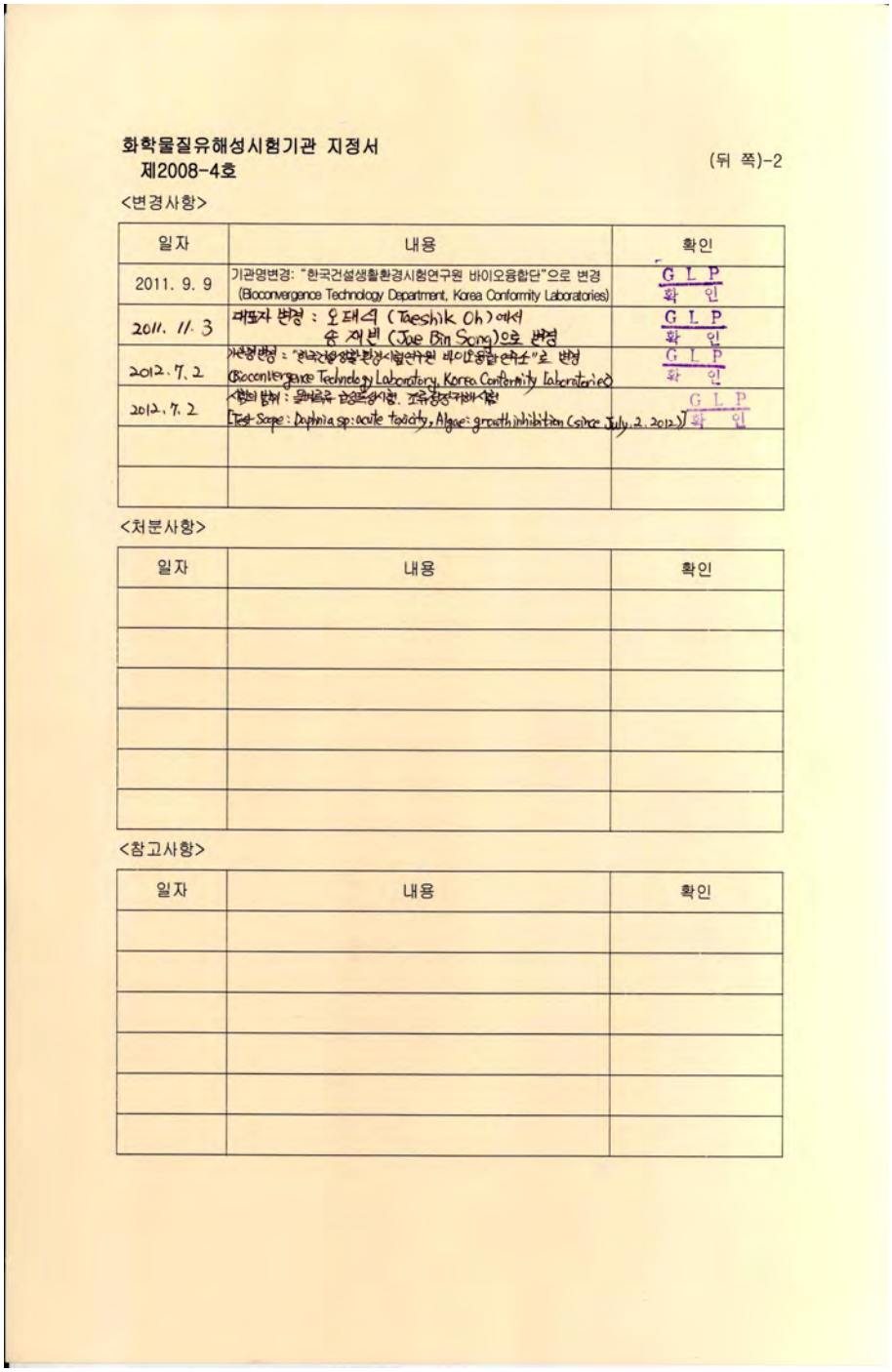
일자	내용	확인
2009. 5. 20	운영책임자 변경 : 유 일 저 (Il-Je Yu) 에서 송 경 석 (Kyung-Seuk Song) 으로 변경	
2009. 11. 16 (구분)	시험의 범위 : 급성경피독성시험, 어류급성독성시험 (완료기간 : 2009년 11월 16일 부터) 끝	
〃 (영문)	Test Scope : Acute dermal toxicity, Fish: acute toxicity (Validation : since Nov. 16, 2009).	
2010. 8. 2	대표자 변경 : 김 창 호 (Chang-ho Kim) 에서 오 태 식 (Taeshik Oh)로 변경	G L P 확 인
2010. 8. 2	기관명 변경 : "한국건설생활환경시험연구원 바이오융합본부"로 변경 *영문명 (Bioconvergence Technology Division, Korea Conformity Laboratories)	G L P 확 인
2011. 9. 9	운영책임자 변경 : 송 경 석 (Kyung-Seuk Song) 에서 이 진 규 (Jin Kyu Lee)으로 변경	G L P 확 인

<처분사항>

일자	내용	확인

<참고사항>

일자	내용	확인
2010. 12.	정기내부평가 결과, GLP규정을 준수하고 있음 (GLP Compliance)	G L P 확 인
2012. 7. 2	정기내부평가 결과, GLP규정을 준수하고 있음 (GLP Compliance)	G L P 확 인



화학물질유해성시험기관 지정서
제2008-4호

(뒤 쪽)-2

<변경사항>

일자	내용	확인
2011. 9. 9	기관명변경: "한국건설생활환경시험연구원 바이오융합단"으로 변경 (Bioconvergence Technology Department, Korea Conformity Laboratories)	GLP 확인
2011. 11. 3	대표자 변경 : 오태식 (Taeshik Oh)에서 송 재빈 (Jae Bin Song)으로 변경	GLP 확인
2012. 7. 2	기관명변경 : "한국건설생활환경시험연구원 바이오융합센터"로 변경 (Bioconvergence Technology Laboratory, Korea Conformity Laboratories)	GLP 확인
2012. 7. 2	시험의 범위 : 물벼룩을 이용한 급성독성시험, 조류생장저해시험 Test Scope : Daphnia sp. acute toxicity, Algae: growth inhibition (since July. 2. 2012.)	GLP 확인

<처분사항>

일자	내용	확인

<참고사항>

일자	내용	확인

Annex 5. Quality assurance statement-Original

신 뢰 성 보 증 확 인 서

시험번호 : GT13-00018

시 험 명 : 포유류 배양세포를 이용한 MWCNT의 염색체이상시험

이 보고서에 기술된 시험을 독립적으로 아래와 같이 시험과정 단계별로 점검하였으며 각 점검결과를 표준작업지침서에 따라 시험책임자와 운영책임자에게 통보 및 보고하였다.

본 시험은 국립환경과학원 고시 제2012-23호 (2012년 08월 22일) '화학물질유해성시험연구기관의 지정 등에 관한 규정' 별표5 제4장 제15항 유전독성시험(염색체이상시험) 및 OECD Guidelines for the Testing of Chemicals No. 473 '*In vitro* Mammalian Chromosome Aberration Test'(Adopted : 21st July 1997)에 따라 수행되었으며, 보고서 작성방법 및 결과의 기술이 시험의 실시과정에서 발생한 시험기초자료를 바탕으로 정확히 반영되었음을 확인하였다.

점 검 내 용	실 시 일	시험책임자에게 통보일	운영책임자에게 보고일
시험계획서 점검	2013. 02. 15	2013. 02. 15	2013. 02. 15
시험물질 및 대조물질	2013. 02. 28	2013. 02. 28	2013. 02. 28
시험계(균주)	2013. 02. 28	2013. 02. 28	2013. 02. 28
배지조제 및 균주접종	2013. 03. 04	2013. 03. 04	2013. 03. 04
시험물질조제	2013. 03. 07	2013. 03. 07	2013. 03. 07
시험물질처리	2013. 03. 07	2013. 03. 07	2013. 03. 07
검체제작 및 검경	2013. 03. 11	2013. 03. 11	2013. 03. 11
시험기초자료	2013. 08. 07	2013. 08. 07	2013. 08. 07
최종보고서 점검	2013. 08. 07	2013. 08. 07	2013. 08. 07

한국건설생활환경시험연구원 바이오융합연구소

신뢰성보증책임자 송 경 석 (인)

2013 년 08 월 07 일

Annex 6. Study personnel-Original

시험관계자 서명

시 험 물 질 조 제	성재혁 시험물질조제분석 책임자	Date 2013. 08. 07
주 시 험 담 당	주효진 주시험 담당자	Date 2013 08 07
세 포 주 보 관	김진식 세포주보관 책임자	Date 2013. 08. 07
자 료 보 관	김효동 자료보관 책임자	Date 2013. 08. 07.